ECTOMYCORRHIZAL FUNGI AND FORESTRY PRACTICE

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Introduction

Much of our understanding on the functions of mycorrhizae has come from research directed towards practical application in forestry. Early in this century, for example, the repeated failures in establishing exotic pine plantations in the tropics and other areas of the world where ectomycorrhizal hosts do not naturally occur clearly demonstrated the dependence of these trees on their fungal symbionts. Only after inoculation with forest soil containing ectomycorrhizal fungus propagules could these trees survive and function properly (23,24). Intensive mycorrhizal research in the past 30 years has increased our understanding of the complex physiology and ecology of ectomycorrhizae and our appreciation of the role of symbiosis (9). Most importantly, this information provides many of the necessary tools and concepts for strengthening forestry programs around the world.

Today, widescale inoculation of forest seedling nurseries with selected ectomycorrhizal fungi appears imminent. Commercial interests in producing pure cultures of ectomycorrhizal fungus inoculum expands the possibilities of worldwide application. The success of these inoculation programs hinges on selection of effective and beneficial fungal symbionts. Little data exists on which of the thousands of possible ectomycorrhizal fungi may be the best candidates for inoculating a particular host species. Other considerations, such as nursery management practices and location of outplanting sites complicate the selection process. New inoculation programs must be strongly research oriented from the outset (34).

In this paper, I briefly review the considerations involved in determining the need for ectomycorrhizal inoculation, the techniques available for inoculation, and some relevant criteria for selecting specific fungi for inoculation. The readers are referred to Trappe (this symposium) for a general discussion of mycorrhizae and to Mikola (23,24) and Trappe (34) for more detailed reviews on applications of ectomycorrhizae in forestry practice.

Forestry Uses of Ectomycorrhizal Inoculation

Ectomycorrhizal host trees must be accompanied by their mycorrhizal fungi to survive when planted in areas lacking suitable ectomycorrhizal fungi. This

has been experienced many times in the introduction of exotic pines into the Southern Hemisphere and tropical islands (1,23). Afforestation attempts in the treeless grasslands of the U. S. and the steppes of Russia have also required inoculation for success (5,21). The more recent work of Schramm (28) and Marx (12) has shown the absolute requirements for ectomycorrhizae for tree establishment on stripmined lands and other severely spoiled sites.

Although successful attempts to inoculate tree seedlings already planted in the field have been reported (1), nursery inoculation is more common. Seedlings inoculated in the nursery can establish a healthy ectomycorrhizal root system before outplanting. The increasing use of soil fumigation to eliminate pests in nurseries makes the mycorrhizal inoculation of nurseries especially critical. Most complete soil fumigants, such as the commonly used methyl bromide-chloropicrin* mixes, thoroughly eradicate ectomycorrhizal fungus populations (4). Tree seedlings lacking ectomycorrhizae show severe nutrient deficiencies early in their first growing season; the deficiencies persist until mycorrhizae are formed (35). Although deleterious to resident fungal populations, nursery fumigation is a necessary tool in present inoculation techniques; competition from the resident ectomycorrhizal fungi is eliminated or reduced as a prerequisite for successful establishment of the selected fungal inoculum.

Newly established nurseries often show mycorrhizal deficiency symptoms, often most severely in nurseries established on heavily fumigated former agricultural land. Trappe and Strand (35) report a striking example of this in the Willamette Valley of Oregon. The first crop of Douglas-fir seedlings exhibited severe phosphorus deficiency, unexpected because preliminary soil analysis indicated no phosphorus deficiency. Subsequent heavy fertilization with phosphates did not alleviate the deficiency. Only after natural inoculation by windblown spores did the seedlings recover and grow vigorously. Substantial dollar losses, work hours wasted, and disruption of reforestation programs resulted.

The millions of containerized seedlings produced in the Pacific Northwest and elsewhere around the world offer another situation where ectomycorrhizal inoculation may be important. Many cultural practices used in raising containerized seedlings--e.g., artificial potting mixes, frequent applications of concentrated soluble fertilizers, and greenhouse rearing--minimize or greatly retard ectomycorrhizal formation. Most of the containerized seedlings we have examined appear vigorous and healthy, but they routinely lack

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*This paper reports work involving pesticides. It does not include recommendations for operational use nor does it imply that uses discussed here are registered unless specifically stated. All uses of pesticides must be registered by appropriate State and Federal Agencies before they can be recommended.

mycorrhizae. We strongly suspect that the more natural mycorrhizal root system will increase survival and initial growth of containerized seedlings upon outplanting. The interactions of inoculation and the cultural practices mentioned above are currently under intensive investigation.

Ectomycorrhizal inoculation of tree seedlings offers promise for increasing reforestation success of suboptimal sites. Inoculation of seedlings destined for cutover lands has sometimes been regarded as unnecessary due to the great abundance of mycorrhizal fungal propagules still present there. We have found this to be true for high quality sites in the Oregon Coast Ranges (Molina and Trappe, unpublished data). We feel, however, that reforestation of drought, heat, or cold stressed habitats will be improved by planting seedlings inoculated with fungi selected specifically for those habitats. In droughty sites, for example, the density of fungal propagules may be low due to the frequent lack of a prolonged fungal fruiting season. The resulting delay in ectomycorrhiza formation on nonmycorrhizal seedlings in these sites may decrease survival or initial growth. Marx et al. (19) have recently reported significant increases in growth and survival of five southern pine species inoculated with the fungal symbiont Pisolithus tinctorius and planted in various reforestation sites in North Carolina and Florida. Much research is still needed on selection of ectomycorrhizal fungi for specific reforestation sites.

Other uses of mycorrhizal inoculation in forestry practices will undoubtedly become evident as we intensify our effort to reforest cutover lands and reclaim spoiled sites.

Techniques of Inoculation Once the need for inoculation is recognized, several methods are available. Spontaneous inoculation from windblown spores may provide sufficient inoculum for nurseries established near stands of ectomycorrhizal host trees. Usually, however, spontaneous inoculation is erratic and gives spotty results. When sowing follows spring fumigation, the chances for adequate natural inoculation the first growing season is slight. In the Pacific Northwest and many other regions, for example, the height of the fungal fruiting season comes with the onset of fall rains and tapers off sharply over winter and into spring; inoculum density of wind disseminated spores is relatively low in the spring. As mentioned before, seedlings will suffer severe mycorrhizal deficiency in their first season until mycorrhizae form.

Relatively few species of ectomycorrhizal fungi commonly inhabit nurseries. Thelephora terrestris, Laccaria laccata, and Inocybe lacera are very common in Douglas-fir nurseries in the Pacific Northwest. These fungi are aggressive and well adapted to the highly fertile, irrigated nursery condition. These nursery fungi may not be well suited to many planting sites.

Another common nursery fungus in temperate region nurseries is an unidentified species that forms ectendomycorrhizae (6). This fungus is rare in natural forests, so its effectiveness is questionable for forest sites.

The four primary sources of artificial inoculum are listed below with their primary disadvantages (24). The advantages of each are then elaborated, but the promising technique of inoculation with pure mycelial cultures is particularly emphasized.

I. Soil inoculum
   1. Bulky, large amounts are needed (10% volume).
   2. Possible introduction of pests and pathogens.
   3. Fungal symbionts unknown.

II. Mycorrhizal nurse seedlings
   1. Possible introduction of pests and pathogens.
   2. Uneven spread of mycorrhizal infection.
   3. Fungal symbionts unknown.

III. Spores and sporocarps
   1. Collection seasonally limiting.
   2. Quantities of sporocarps limiting.
   3. Much hand labor in sporocarp collection.
   4. Long-term storage requirements unknown.

IV. Pure cultures
   1. Difficulty in isolating some ectomycorrhizal fungi.
   2. Growth of most fungi slow in pure culture.
   3. Production of sufficient inoculum time consuming and expensive.
   4. Conditions for survival of fungal inoculum in the soil poorly known.
   5. Best fungi to use uncertain, i.e., proven effective and beneficial fungal symbionts needed.

The most commonly used and probably the most reliable source of ectomycorrhizal inoculum is soil taken from beneath ectomycorrhizal hosts (24). The technique simply involves the incorporation of roughly 10% by volume of soil inoculum into the nursery beds prior to sowing or transplanting. Soil inoculum can also be added to the planting hole when the seedlings are outplanted. Soil inoculation has been instrumental in the establishment of exotic pine plantations in the Southern Hemisphere and continues as a regular practice there today (23).

A major disadvantage of soil inoculation is the relatively large amounts of soil needed and transportation problems in moving it long distances. Within individual nurseries, however, inoculation of new or fumigated beds with soil from established beds is feasible. A possible serious problem

with soil inoculum is the introduction of pathogens and other pests. Nursery managers may be reluctant to incorporate nonfumigated soil into fumigated beds. Fortunately, this widely used inoculation technique has seldom been found to create pathogen problems (34). Soil inoculation remains a reliable method of eliminating mycorrhizal deficiencies and deserves further research attention.

Planting mycorrhizal "nurse" seedlings into nursery beds as a source of the fungi for neighboring young seedlings has been successful (23,24). The mycorrhizal infection, however, tends to spread slowly and unevenly. The major disadvantages of this method parallel those of soil inoculation--there remains the possibility of introducing unwanted pathogens and other pests; and with both techniques, the identities and effectiveness of the introduced fungi are unknown.

Spores or crushed sporocarps have been used occasionally as inoculum, usually in small experiments. Some investigators have reported good success with this technique (2,13,29,30). Theoretically, the use of spores would most closely imitate natural inoculation. Practical application is limited, however, by the generally short season for collecting sporocarps in quantity. In some regions, adverse weather may often even eliminate the collecting season. Also, in many regions the fungi fruit in fall but the nurseries fumigate and sow seeds in the spring. Spore inoculum then must be stored over winter, but little is known of the storage requirements of fungal spores. The Gastromycetes (puffballs), with abundant spore masses, offer better sources of large numbers of spores than the gilled fungi. Asexual spores and sclerotia are further sources of inoculum (8,31). As more becomes known about the basic biology of these fungal propagules, their use in nursery inoculation may become more practical.

The final type of inoculum is pure cultures of ectomycorrhizal fungi. Although many difficulties remain in using this source, techniques for wide scale application are now being developed.

A pure culture of a specific fungus must first be isolated either from the fruiting body or the ectomycorrhiza itself; occasionally, isolates can be obtained from spores or surface sterilized rhizomorphs or sclerotia. The ubiquitious ectomycorrhizal fungus Cenococcum geophilum (=C. graniforme) is easily isolated from its hard black sclerotia (33). Isolation from sporocarps is, however, easiest for most fungi and permits identification of the species. Isolations from ectomycorrhizae or rhizomorphs are more difficult, and the species often cannot be identified (37). Unfortunately, many ectomycorrhizal fungi have never been isolated or grow extremely slow in culture. Still, many do grow well in culture. We find that most species of Suillus, Hebeloma, Laccaria, Amanita, Rhizopogon, and Pisolithus grow rapidly in culture; so much research attention has been devoted to these species. As improved isolation and culturing procedures are developed, many other fungi can be considered.

Although pure culture inoculation has been restricted primarily to small scale experiments, Moser (25,27) reported 20 years ago the successful inoculation of nursery beds of Pinus cembra in Austria with pure cultures of Suillus plorans. Vozzo and Hacskaylo (36) later used Moser's methods to inoculate pine seedlings in Puerto Rico. Marx and Bryan (17) have further refined Moser's technique and report excellent results in inoculating nursery beds with Pisolithus tinctorius. They grow the fungus 3 to 4 months in 2-liter fruit jars containing a sterilized peatmoss-vermiculite substrate moistened with modified Melin-Norkrans nutrient solution (10). After the fungus completely penetrates the substrate, the inoculum is removed from the jars, thoroughly leached with cool running tap water to remove unassimilated nutrients, dried at low heat to about 20% moisture, and placed in cold storage until used. To inoculate nursery beds, the dried inoculum is spread on the soil surface at the rate of about 100 cm^3 per square foot and chopped with a hand spade 3 to 4 inches into the soil. The bed surface is then smoothed and machine sown. Thorough fumigation of the nursery soil, preferably with a methyl bromide-chloropicrin mix, is critically needed before inoculation to reduce competition and antagonism by other soil organisms.

Inoculation of containerized seedlings with pure cultures also holds great promise (15), (Trappe and Molina unpublished data, see Figure 1). A peatmoss-vermiculite mix is used both as an inoculum substrate and container potting mix, so the inoculum is easily incorporated when containers are filled.

The logistics of producing massive quantities of inoculum presently limits wide scale use of pure culture inoculum. Clearly, producing nursery inoculum in 2-liter fruit jars is impractical. Large scale production methods are now being developed, however, by a pharmaceutical firm, Abbott Laboratories.* A nationwide evaluation of this commercially-produced inoculum in nursery beds and containers is currently underway. Industry representatives and mycorrhiza researchers are optimistic that commercial inoculum will be effective and soon available on the market.

Selection of Fungi for Inoculation The promising outlook for pure culture inoculation raises still another important question: Which fungus is best for a particular host or habitat? The need for information on the effectiveness of the various mycorrhizal fungi

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*The use of trade, firm, or corporation names in this paper is for the information and convenience of the reader. Such use does not constitute an official endorsement by the U. S. Department of Agriculture of any product or service to the exclusion of others which may be suitable.

on different host species has been repeatedly emphasized in the literature, yet little data exists. Thousands of ectomycorrhizal fungi and numerous hosts have been reported (32), so the careful selection of the best fungi for particular hosts is critical.

Many important criteria must be considered when selecting fungus candidates for nursery inoculation. The major criteria are:

1. Ease of isolation.
2. Growth rate in pure culture.
3. Effectiveness as inoculum.
4. Effects on host growth and vigor.
5. Ecological adaptions and ecotypic variation.
6. Interaction with other microorganisms.
7. Host specificity.

Other criteria may be added for special circumstances. It must be stressed at this point that the many species and ecotypes of fungi are closely adapted to their particular habitats, and so each fungal isolate must be tested on its own merits. Careful experimentation and good record keeping are essential during initial attempts with each isolate to determine how well it meets the criteria as compared to alternative isolates.

Criteria 1 and 2 are basic to all the others: one must first be able to isolate the particular fungus and grow it reasonably well in culture. Following the experience of Moser (26), we routinely isolate in the field using a small portable hood to reduce air movement (see Figure 2). For isolation, we have had best results using small test tube agar slants containing either Melin-Norkrans agar as modified by Marx (10) or potato dextrose agar.

Relatively fast growing fungi are generally preferred for inoculation because of their short incubation period. Unfortunately, many otherwise desirable ectomycorrhizal fungi grow slowly. As the physiological growth requirements of mycorrhizal fungi become better understood, growth of the slow-growers may be improved for use in inoculation. Fungi that do not grow or grow slowly in culture may be highly specialized in their symbiotic relationship to the host and benefit their host greatly. Clearly, further study of these recalcitrant fungi is needed.

Criteria 3 and 4 are next considered in the selection process. After the fungal inoculum has been prepared, its effectiveness must be determined. Feeder roots susceptible to mycorrhizal colonization do not form on seedlings until 6 to 8 weeks after seed germination (7). During this period the vermiculite particles are believed to provide a protective niche for the naked mycelium. Survival and effectiveness of the inoculum is determined by examining roots for ectomycorrhizal formation. Roots should be sampled periodically during the first growing season. The numbers and kinds of other native mycorrhizal types should also be noted to assess effectiveness of the soil fumigation and the competitive ability of the inoculated fungus. All nursery cultural practices should be carefully monitored and recorded for future reference.

The most crucial criterion in the selection process deals with the benefits the host derives from inoculation with a specific fungal symbiont. Growth differences between inoculated seedlings and uninoculated controls in height, top and root weights, and stem caliper must be compared. Marked improvement in nursery seedlings can be expected by effective inoculation with a highly beneficial fungus. Because the seedlings are raised for forestation purposes, however, the critical test is survival and growth after outplanting as compared to normally produced nursery stock. Survival and growth data must be collected over the first 3 years. Nurserymen will not want to expend the effort to inoculate seedlings unless it significantly improves nursery production or field performance.

Criterion 5 deals with fungal physiology with special reference to ecological adaptability. Field observations as well as laboratory tests are important. Data should be recorded on the ecological range of the fungus as well as specific habitat types in which it is found. Environmental conditions of outplanting sites also need consideration. Planting sites characterized by drought or temperature extremes are commonly difficult to reforest. In mine spoils, soil toxicity is a major problem. Temperature and moisture requirements of the fungus can easily be estimated from simple laboratory tests (34). Special research emphasis should be placed on the ecotypic variation displayed within fungal species; each fungus strain must be tested on its own merit (34). Our working hypothesis is that fungi already adapted to conditions similar to the planting sites should be the primary candidates for inoculation. Trappe (34) states "In essence, the provenance of the fungus should be considered along with the provenance of tree seed."

Ectomycorrhizal fungi protect host roots to varying degrees against certain pathogens (11). Although criterion 6 has never been applied in nursery inoculation, it has potential. Nurseries with continuing root pathogen problems may wish to introduce mycorrhizal fungi selected for ability to protect seedling roots from disease.

The final criterion is host specificity. Many ectomycorrhizal fungi exhibit wide host ranges: Amanita muscaria, Boletus edulis, Laccaria laccata, Pisolithus tinctorius and Cenococcum geophilum, to mention a few. Others are more restricted. Some are known only to fruit in association with a single host or genera of hosts. The association of Suillus grevillei with Larix species and Leccinum scabrum with Betula species are two commonly cited examples. The genus Pinus appears to have its select group of "pine" mycorrhizal fungi. Douglas-fir, Pseudotsuga menziesii, has many mycorrhizal fungi common only to its distribution. Precise data of this nature is very important if we plan to inoculate a wide range of tree species, especially in regions where many different host genera are raised commercially. The Pacific Northwest, for example, contains at least 16 native genera of ectomycorrhizal hosts totaling over 60 different species (Table 1). At least a third of these species are raised in commercially bare-root and container nurseries. A single nursery may raise 10 different tree species. Should many different fungi be inoculated to satisfy the different hosts or is it better to inoculate with one fungus capable of infecting them all? Mikola (24) believes that, due to its more specialized relationship with a particular host, a host-specific fungus would benefit its host more than would a broad range fungus. This hypothesis warrants further research, especially with the development of wide scale, pure culture inoculation.

Pure culture synthesis of mycorrhizae as developed by Melin (22) and modified by Hacskaylo (3) and others provides good evidence on host specificity. Seedlings are raised aseptically in two-membered culture with an introduced mycorrhizal fungus. With large glass test tubes for the chambers (Figure 3), numerous combinations of host species and fungi can be readily assessed in 3 to 6 months when the seedlings are harvested. Often, mycorrhiza formation can be observed directly through the glass wall of the tube (see Figures 4-10).

The Pisolithus Story--A Case in Point A review of mycorrhizal inoculation would not be complete without mentioning the intensive research on the fungus Pisolithus tinctorius being conducted by Dr. Donald Marx and co-workers at the U. S. Forest Service Institute for Mycorrhizal Research and Development, Athens, Georgia. Their successful inoculation program has gathered the support of both the research community and industry and has brought mycorrhizal application in forestry to the forefront of mycorrhizal research. Although focusing on one fungal symbiont, P. tinctorius, their underlying hypothesis is that growth and survival of seedlings can be significantly improved by inoculations with specific mycorrhizal fungi. A brief summary of their work follows with special emphasis on their integrated use of many of the concepts presented in this paper.

Schramm (28) reported the extensive development of Pisolithus tinctorius ectomycorrhizae and sporocarps associated with pine roots growing on anthracite mining wastes in Pennsylvania. P. Tinctorius was often the pioneering

mycorrhizal symbiont on the young, most vigorous pine seedlings. Realizing that the extremely high soil temperatures reported by Schramm might limit fungal symbionts to a few adapted species, Marx et al (20) explored the temperature-growth interactions of P. tinctorius. They found that it formed more ectomycorrhizae with Pinus taeda seedlings at 34°C. than at lower temperatures; mycelial cultures grew at temperatures as high as 40°C. Marx and Bryan (16) later found that aseptically grown Pinus taeda seedlings infected with P. tinctorius survived and grew as well at 40° as at 24°C; comparative nonmycorrhizal seedlings and those infected with the fungal symbiont Thelephora terrestris had less survival and did not grow at 40°C. Clearly, the adaption to higher temperatures was a major factor in allowing P. tinctorius to invade the coal wastes.

Realizing the practical significance of these results, Marx (12) and co-workers extensively surveyed strip-mined lands for the presence of Pisolithus tinctorius. They found P. tinctorius to be the dominant, and often the only ectomycorrhizal fungus of pine roots growing on coal wastes in Indiana, Pennsylvania, Ohio, West Virginia, Virginia, Kentucky, Tennessee, and Alabama and on kaolin spoils in Georgia.

These results prompted extensive investigation of ways to inoculate and establish Pisolithus tinctorius ectomycorrhizae on roots of pine seedlings destined for outplanting on mine spoils. Marx and Bryan (17) developed techniques as previously described for preparing pure culture inoculum and inoculating nursery soil with P. tinctorius. They report excellent success in establishing P. tinctorius in the nursery with doubled growth of inoculated seedlings over uninoculated controls (18). Inoculation with P. tinctorius basidiospores has also succeeded (13,18); billions of basidiospores can often be collected easily from this puffball which produces large sporocarps. More importantly, Marx (12) reports significantly increased survival and growth of P. tinctorius-inoculated seedlings on mine spoils. Many of these sites had a history of repeated failures of pine plantations. P. tinctorius ectomycorrhizal colonization can also increase survival and growth of southern pines on routine reforestation sites (19).

Development of the Pisolithus tinctorius inoculation program included many of the selective criteria discussed previously. P. tinctorius is easily isolated from sporocarps and grows rapidly in culture. Inoculation with pure cultures of vegetative mycelium as well as basidiospores often completely colonizes the entire feeder root system. Overall host response and performance in both nursery and plantation are excellent. Both field and laboratory studies emphasize the ecological adaptiveness of P. tinctorius to stressful sites, including tolerance to high soil temperatures, moisture stress, and soil toxicity. Finally, P. tinctorius is distributed worldwide and forms ectomycorrhizae with over 48 species of trees (14); worldwide use of this highly beneficial fungus is quite possible.

These impressive results and the concepts they represent has stimulated research inoculation programs around the world. Feasibility of utilizing commercially produced Pisolithus tinctorius inoculum as well as other fungal symbionts in nurseries throughout the U. S. is now being studied. Clearly, with the increased demand for and dwindling supply of wood resources, regeneration of cutover lands and establishment of man-made forests is of utmost priority. Inoculation with highly beneficial mycorrhizal fungi specifically selected for certain traits can enormously increase the chances of meeting this priority.

TABLE 1. GENERA OF NATIVE ECTOMYCORRHIZAL HOSTS IN THE PACIFIC NORTHWEST

Genus	No. of species
Pseudotsuga	1
Tsuga	2
Picea	2
Abies	6
Pinus	7
Alnus	4
Populus	2
Larix	2
Quercus	2
Castanopsis	2
Corylus	1
Arbutus	1
Arctostaphylos	4
Salix	30
Betula	5
Cercocarpus	2

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LIST OF COLOR FIGURES